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Chapter Sixty-Four

THE IDENTIFICATION OF A *LEGIONELLA PNEUMOPHILA* TOXIN WITH *IN VIVO* LETHALITY

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Only 7 months intervened between the initial reports of the dramatic outbreak of Legionnaires' disease in Philadelphia and the discovery of the etiologic agent now termed *Legionella pneumophila* by Fraser[1] and McDade[2] and their colleagues. Legionnaires' disease has been recognized in 43 states, the District of Columbia,[3] Canada, Australia, England, Israel, Scotland, Denmark, Spain, The Netherlands, Italy, and New Zealand.[3] The largest outbreak of legionellosis outside the United States occurred in Västerås, Sweden, from August 28 to September 21, 1979 and involved 67 cases.[4]

Two distinct clinical syndromes are now recognized to be associated with *L. pneumophila*. The first, termed Legionnaires' disease, is a rapidly progressive and fulminant pneumonia showing patchy infiltrates on x-ray film that may have an interstitial or consolidated appearance and progress to nodular unilateral or bilateral consolidation. This pneumonic form has a 16 percent lethality in normal individuals and a 54 percent lethality in immunologically compromised patients. Death is associated with either respiratory failure or shock. The second syndrome presents as a nonpneumonic, nonlethal debilitating flu-like illness popularity termed "Pontiac fever." What determines whether *L. pneumophila* will cause Legionnaires' disease or Pontiac fever is not characterized [5] It has been suggested that Pontiac fever might result from a large dose of nontoxigenic organisms.

In 1978 Friedman[6] and Winn and colleagues[7] postulated that a toxin or toxins might be involved in the pathogenesis of Legionnaires' disease for two dissimilar reasons. Dr. Friedman noted the disease generally involved the lungs, kidneys, and central nervous system. Although the pathogenesis of the multiple organ involvement was unclear, he offered as a possible explanation the production of a toxin by *L. pneumophila*. Winn reviewed 14 fatal cases

from the 1977 outbreak in Vermont and suggested that the production of a cytolytic toxin by *L. pneumophila* could explain the lysis of the inflammatory exudate and the infarct-like necrosis of alveolar walls seen in several cases.[7] Subsequently, Wong and colleagues[8] of CDC reported on the endotoxin properties of *L. pneumophila*. The fact that this gram-negative rod possessed lipopolysaccharides (LPS) that reacted positively with limulus lysate was not unexpected. However, these investigators demonstrated that *Legionella* LPS was biochemically and functionally different from classic endotoxins. It lacked hydroxy fatty acids, was minimally inhibited by treatment with polymyxin B, and was minimally potentiated by dactinomycin. The weak capacities of *Legionella* LPS in inducing heparin-precipitable proteins, Schwartzman reactions, and pyrogenicity also demonstrated that the organism was comparatively weak in *in vivo* "endotoxicity."

Baine and colleagues[9] reported hemolytic activity found in sterile filtrates of allantoic fluid of eggs in which *Legionella* had grown. This activity was most pronounced against guinea pig red blood cells and only slightly with human red blood cells. Since certain exotoxins can cause hemolysis, it was suggested that this activity might be an exotoxin. In a subsequent article Baine and colleagues[10] cautioned that the hemolytic activity might not be due to the presence of bacterial hemolysins. Recently, Müller demonstrated various proteolytic enzymes associated with *L. pneumophila* and suggested that they might play a pathogenic role.[11] Friedman and colleagues[12] have recently identified a low molecular weight protein found in *L. pneumophila* culture filtrate material cytotoxic for Chinese hamster ovary cells. Our interest in the possible toxin or toxins of *L. pneumophila* extends back to our own reported findings that AKR/J mice challenged with lethal doses of *L. pneumophila* displayed a histologic pattern of randomly scattered hepatocellular necrosis involving individual cells along with larger foci of coagulative necrosis and a scattering of hepatocytes with coarse vesiculated cytoplasm compatible with the effects caused by a toxin.[13]

This chapter reports the demonstration of a low molecular weight protein toxin derived from a cell-free extract of sonicated *L. pneumophila*. The antigenic relationship of this toxin with a similar extract, obtained from a newly discovered, but genetically unrelated, gram-negative rod termed the Pittsburgh pneumonia agent, is also shown.

MATERIALS AND METHODS

Toxin Production

Approximately 10 grams (gm) of *L. pneumophila* Washington, Chicago, or Atlanta strain) were harvested from the surface of modified Müeller-Hinton plates incubated at 35° C in 5 percent CO_2 for 4 days. If the organisms were washed in normal saline, the concentrated wash material was not lethal for AKR/J mice. Conversely, a lethal, cell-free extract can be obtained in the following fashion:

Ten-gram aliquots of washed pellets are sonicated at 5° C three times for 2-minute intervals using a Fisher Sonic Dismembrator Model 300. The disrupted cells are resuspended in a total volume of 40 milliliters (ml) of normal saline and centrifuged at 1500 × G for 30 minutes at 5° C. The supernatant material is filtered through 0.45 micrometer (μm) and then through 0.22-μm

filters. The toxicity of each crude supernatant preparation is assayed by its LD_{50} in AKR/J within a 24-hour period.

Molecular Weight Estimation

A Sephadex G-50 column (1.5 × 45 cm) was calibrated according to the method of Andrews[14] with the use of chymotrypsinogen A, ribonuclease A, aprotinin, and bacitracin as markers.

Preparation of Antiserum

One-milligram amounts of protein from *Legionella* acid supernatant were emulsified in 1 ml of incomplete Freund's adjuvant and injected either intramuscularly into goats or into the foot pads of rabbits. The animals were boostered with the same material after 28 days; 7 days later they were bled for antibodies. Prebleed serum had been obtained as a control.

RESULTS

Acid Precipitation of Crude Toxin

Hydrochloric acid (1 N) was slowly added to rapidly stirred crude toxin until pH 3.5 was obtained. This opalescent mixture was then recentrifuged at 1500 X G. After membrane filtration, the toxic activity was found to be localized to the acid supernatant, and the pellet was discarded. This step resulted in approximately a 70 percent reduction in total protein.

Bio Gel A 5m Gel Filtration

Aliquots of acid supernatant from the previous step were applied to a Bio Gel A 5m column and eluted with 0.1 molar (M) phosphate buffered saline, pH 7.4. The toxic activity appeared with the void volume. The first peak constituted approximately 5 to 10 percent of the total protein applied to the column depending upon the individual acid supernatant preparation. There were no observable differences in lethality between toxin derived from any of the three *Legionella* strains (Atlanta, Chicago, Washington) (Fig. 1).

The composition of the two A 5m peaks changed with individual lot samples, but had approximately the proportionate composition (Table 1). Although 0.5 ml of the first peak readily kills mice within 18 hours, as much as 6 ml of the second peak does not. The total amount of 2-keto-3-deoxyoctonate (KDO), carbohydrate, and protein delivered to the animals receiving the 6 ml from the second peak exceeds the amount in the lethal first peak. In support of the work of Wong and colleagues[8] concerning the relative lack of

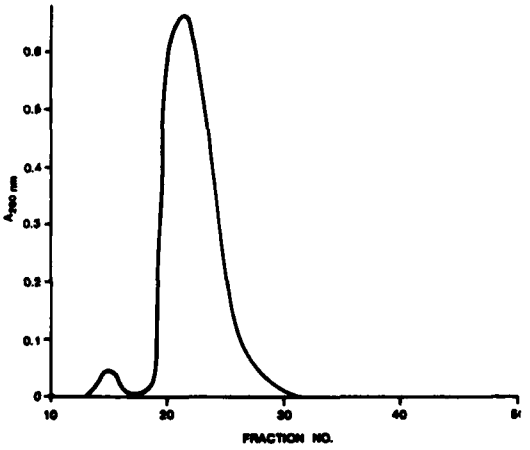

FIGURE 1. Elution pattern of *Legionella* acid supernatant toxin preparation on a Bio Gel A 5m column (5 x 11 cm) equilibrated with 0.1 M phosphate buffered saline. The sample volumes were approximately 10 ml; 12-ml fractions were collected. The toxin activities were determined by intraperitoneal inoculation into AKR/J mice.

TABLE 1 Composition of the two peaks

COMPONENT	FIRST PEAK (μg/ml)	SECOND PEAK (μg/ml)
Protein	50	325
Carbohydrate	7	23
KDO	0.8	0.6

endotoxicity of *L. pneumophila* LPS, we have injected hot phenol-water extracted *Legionella* endotoxin, containing as much as 15 μg of KDO material, into mice without lethal effects.

Preparative Isotachophoresis.

Preparative isotachophoresis was performed in a vertical column electrophoresis apparatus (LKB 7900 Uniphor) using buffers and 4.5 percent polyacrylamide gel supporting medium according to the method of Svendsen and Rose.[15] Single buffered gel columns (cross-sectional area 3.48 cm^2, length 14 cm) were used and the Ampholine carrier (pH 4 to 9) was mixed with the sample. Dearated TRIS-phosphate, pH 7.05, was used both in the lower electrode (anode) chamber and the elution buffer. The terminating buffer, TRIS-ε-aminocaproate (TRIS-EACA), pH 8.45, was used in the upper electrode (cathode) chamber and in the column above the gel and sample.

A sample of 7 to 10 ml of the first A 5m acid supernatant peak containing 50 μg of protein/ml was mixed with 1 ml of glycerol to increase viscosity. The mixture was then layered on top of the gel by tubing inserted through the top layer of TRIS-EACA buffer, which was held within a few millimeters of the gel surface. The experiments were performed using a constant power source (LKB 2127). A current of 4 milliamperes (mA) and a starting voltage of 1.5 kilovolts (kV) were applied in every run. Cooling water was ambient and usually ranged between 20 and 28° C. The elution rate was 18 ml/hour. The eluate was recovered in 12-ml aliquots on a fraction collector. UV absorption at 280 nanometers (nm) of each fraction was read on a Beckman DBG spectrophotometer (Fig. 2).

Molecular Weight

Toxin eluted as one symmetric peak. The molecular weight of the toxic moiety, isolated by preparative isotachophoresis, was estimated to be approximately 3400 daltons (Fig. 3).

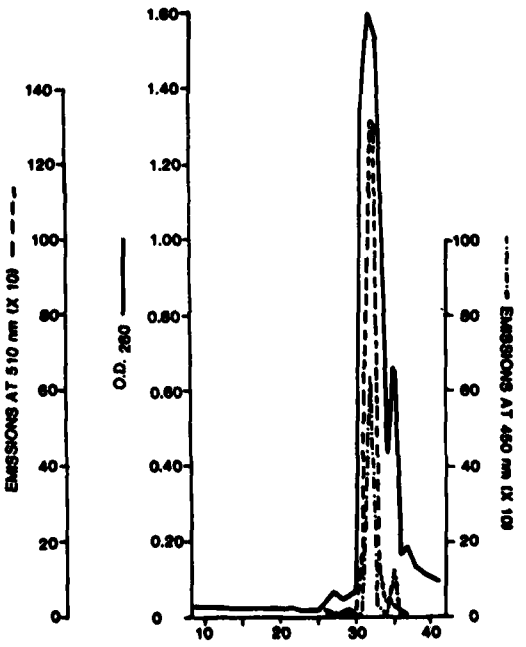

FIGURE 2. Preparative isotachophoresis run from a Uniphor column in which a semipurified *Legionella* toxin was separated according to Svendsen and Rose.[10]

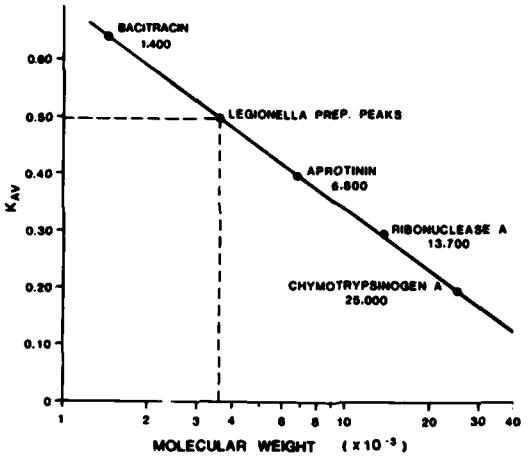

FIGURE 3. Determination of *Legionella* toxin on Sephadex G 50 column (1.5 x 45.0 cm) by extrapolation.

FIGURE 4. *Legionella* toxin examined by immunoelectrophoresis. Antigen in *well A* is *Legionella* acid supernatant. Toxin is in *well B* (preparative isotachophore peak of acid supernatant). Anode is to the right. The rabbit antibodies in the central trough were directed against *Legionella* acid supernatant. A Coomassie brilliant blue R stain was used.

Immunologic Reactivity of Preparative Isotachophoresis-Derived Toxin

The relationship of *Legionella* acid supernatant and the preparative isotachophore peak is depicted in Figure 4. A single precipitin band identifies the *Legionella* preparative isotachophoresis peak.

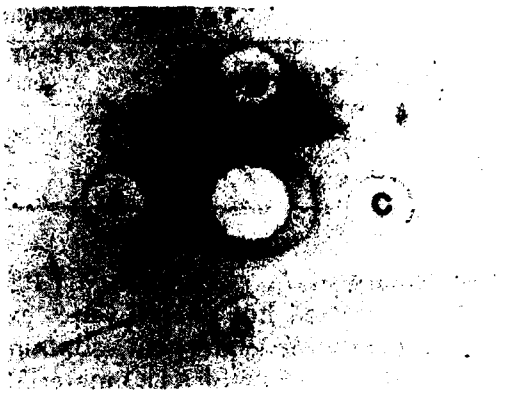

FIGURE 5. Immunodiffusion plate *wells A* and *B* contain *Legionella* acid supernatant and toxin (preparative isotachophore peak), respectively; *wells C* and *D* contain PPA acid supernatant and PPA preparative isotachophore peak. The *center well* contains antiserum to *Legionella* acid supernatant. A line of identity joins a common antigen in the four outer wells.

Antigenic Relationship of Legionella Toxin and a Similar Protein Derived from the Pittsburgh Pneumonia Agent

Recently Pasculle and colleagues[16] and Myerowitz and colleagues[17] reported the first isolation of a new gram-negative bacteria called the "Pittsburgh pneumonia agent" (PPA). PPA resembles *L. pneumophila* in pathogenicity, growth requirements, and composition of fatty acids. The two bacteria differ in their genetic relatedness, reported antigenic composition, and colonial morphology. Using techniques identical to the ones already described, we have recently been able to show that PPA acid supernatant preparations and preparative isotacho-phore peaks are also able to kill AKR/J mice by intraperitoneal inoculation. Immunodiffusion studies show that *Legionella* acid supernatant and toxin share a common antigen with PPA acid supernatant and its preparative isotachophore peak, which also has the same molecular weight (3400 daltons) as *Legionella* toxin (Fig. 5).

DISCUSSION

We have demonstrated a 3400-dalton protein isolated from a cell-free sonicate of *L. pneumophila* that maintains its *in vivo* lethality for AKR/J mice through a variety of isolation procedures, including acid precipitation, gel filtration, and preparative isotachophoresis. The preparative isotachophoresis step allows the nondestructive dissociation of a high molecular weight protein entity (>5 million daltons) isolated by A 5m gel filtration and concentrates the toxic activity as a single low molecular protein. Although *L. pneumophila* and the PPA are genetically unrelated by DNA homology techniques, they cause similar clinical illnesses. We have shown an antigenic relationship between cell-free extracts of both these organisms that are lethal for AKR/J mice *in vivo* and believe that this toxin may play at least a partial role in the pathogenesis of the disease.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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